Engineering Suicide Gene Approaches to Improve Chemotherapeutic Response in Glioblastoma

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Engineering Suicide Gene Approaches to Improve Chemotherapeutic Response in Glioblastoma

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Abstract

Glioblastoma is the most common brain cancer in adults and is uniformly lethal. Existing therapies, namely surgery followed by temozolomide chemotherapy and radiation, fail to prevent disease recurrence and new treatment approaches are urgently needed. Suicide genes are a potentially promising approach for glioblastoma wherein viruses are used to transduce tumor cells to express a foreign protein that converts a prodrug into a toxic product, resulting in cell death. Utilizing the herpes simplex virus thymidine kinase (HSVtk) suicide gene, we designed novel fusion proteins we hypothesized to be post-translationally stabilized following temozolomide treatment. HSVtk was fused to PEST sequences (domains rich in proline, glutamate, serine, and threonine that control protein turnover) predicted to be stabilized via phosphorylation by p38, a kinase activated by the stress of temozolomide-mediated DNA damage. Suicide gene constructs were retrovirally transduced into human glioblastoma cell lines and analyzed for their stability and specificity to p38 by immunoblotting and flow cytometry. The results of this work demonstrate the potential utility of combining a suicide gene with traditional chemotherapy.

Keywords: suicide gene, glioblastoma, kinase-dependent stability

Introduction

Glioblastoma is the most common and the deadliest brain cancer found in adults (1). Glioblastoma cells originate from support cells that exist in the brain (5). Cells have an innate ability to fix DNA when mutations occur; however, cells become cancerous because of gene mutations that cause uncontrolled proliferation which cannot be fixed. The current front-line therapy for glioblastoma is resecting the majority of the tumor, followed by radiotherapy and chemotherapy, in particular temozolomide (TMZ). These therapies have allowed for some increase in survival rates, however the 5-year survival rate is about 7% (6). These treatment methods are decades old and there have been few new treatment methods that have come about since then. Glioblastoma is a difficult cancer to treat due to the heterogeneity in the cellular pathways as well as acquired resistance. Understanding and targeting the pathways used by glioblastoma cells to develop therapeutic resistance could help increase patient survival. Gene therapy represents a possible oblique approach to therapy that can synergize with existing modalities and allows for a novel treatment method which could increase the patient survivability rate.

There have been previous attempts at creating targeted inhibitor therapies for glioblastoma treatment, however they have lacked the ability to improve patient survival rates (7). Suicide genes are a potentially promising approach for glioblastoma wherein viruses are used to transduce tumor cells to express a foreign protein that converts a prodrug into a toxic product, resulting in cell death. The focus of our project is to engineer a suicide gene that cooperates with TMZ chemotherapy in glioblastoma. Our design is based on herpes simplex virus thymidine kinase (HSVtk), which converts the prodrug ganciclovir (GCV) to a toxic phosphorylated form. Once the GCV is phosphorylated it can incorporate

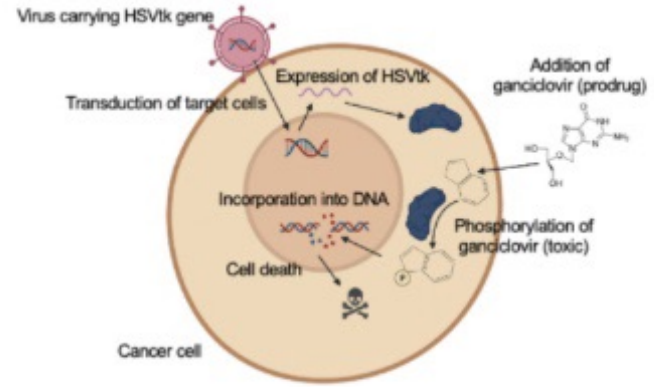

Figure 1. Schematic of the process that causes stabilization of p38 to cause phosphorylation of GCV.

into the new forming DNA and prevent the elongation step, driving apoptosis. A retroviral delivery system is used, as these vectors target dividing cells and would allow protection of healthy brain tissue (Figure 1) (7). Our lab previously designed a suicide gene using HSVtk fused to a portion of the Fos-related-antigen 1 (FRA1) transcription factor, which is phosphorylated by ERK, a highly expressed oncogenic kinase present in glioblastoma cells (4). The HSVtk-FRA1 protein degraded when ERK was inhibited and stabilized when ERK was present which allowed for selective stabilization of the suicide gene (Figure 2) (8). Here, we created versions of HSVtk fused to PEST sequences (domains rich in proline, glutamate, serine, and threonine that control protein turnover) predicted to be stabilized by p38, a kinase activated by the stress of temozolomide-mediated DNA damage. A key difference in the previous ERK stabilized design and the design we have engineered is that our design has post-translational control using stress-driven signaling to lead to stabilization of the suicide gene. The ability of these next-generation suicide genes to augment the effects of chemotherapy and the molecular mechanism involved were studied in glioblastoma cells using immunoblotting, immunofluorescence microscopy, and flow cytometry. Through the combination of chemotherapy drugs we will be targetting post-translational control of the suicide gene.

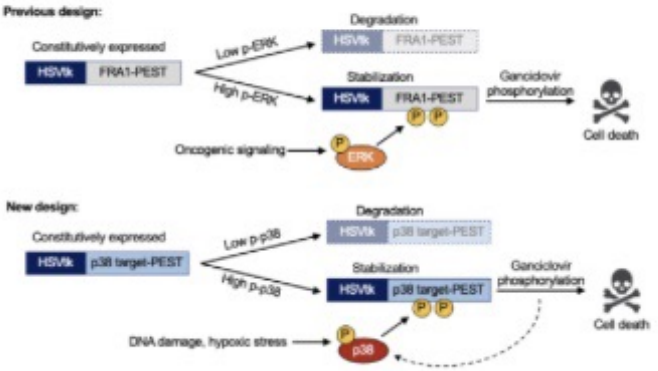

Figure 2. Schematic showing the signaling pathway targeted by the previous design as well as the new proposed pathway.

Results

Suicide Gene Design

Suicide gene therapies are a potentially promising therapeutic option for tumors that otherwise do not respond well to therapy. In a suicide gene therapy approach, tumor cells are virally transduced with a gene that causes cell death in the presence of a normally innocuous prodrug. In the system shown in Figure 2, herpes simplex virus thymidine kinase (HSVtk) phosphorylates the prodrug ganciclovir (GCV). Phosphorylated GCV is incorporated into DNA, preventing strand elongation and promoting apoptosis in transduced cancer cells.

The suicide gene that we will be working with uses an engineered HSVtk fusion protein hypothesized to be stabilized by p38 phosphorylation (9). p38 is overexpressed in glioblastoma cells than normal brain cells which will allow for targeted therapy. p38 activity is dependent on stress, so the greater the stress in the cellular environment the greater the p38 activity will be (9). In particular, DNA damage caused by TMZ chemotherapy is a strong inducer of p38 activity. Prior work has shown that p38 activity can be induced with the use of transforming growth factor-beta (TGFβ), tumor necrosis factor alpha (TNFα), and TMZ. It was shown that p38 activation was most persistent in the TMZ case which would be useful in causing stabilization of the suicide gene (9).

We have designed four HSVtk fusion proteins (Figure 3), with PEST domains that were found using literature and

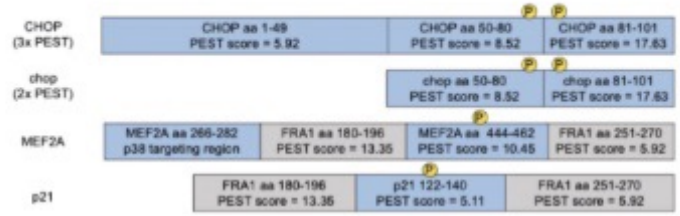

Figure 3. Schematics of each design are shown, with the predicted PEST domains and relative position of serine residues demonstrated in the literature to be phosphorylated by p38 (yellow circles). Sequences shown in blue were taken from proteins primarily phosphorylated by p38, while domains in gray were taken from the original FRA1-PEST design. The ERK recognition sequence was removed from these designs to improve specificity toward p38.

an online PEST domain identifying software created by EMBL-EBI. The four fusion proteins utilized sequences from C/EBP homologous protein (CHOP), Myocyte Enhancer Factor 2A (MEF2A), p21, and/or FRA1 (Figure 3). PEST scores were calculated using the equation: PEST score = 0.55 * DEPST - 0.5 * hydrophobicity index (10). DEPST is a mass fraction of critical amino acids in a certain sequence of amino acids. A high PEST score, greater than 5, means there is a higher predicted rate of protein turnover. In each construct there was at least one domain that had a PEST score of greater than 5 and one serine that was reported to be phosphorylated by p38.

Figure 4. Schematic of the suicide gene cassette. The PEST domains are the four designs outlined in Figure 3.

The fusion genes were cloned into a retroviral pMSCV expression vector. The gene contains an N-terminal FLAG tag to allow for quantification of the protein through the use of immunoblotting or immunofluorescence microscopy. 3' to the gene cassette there is also an internal ribosomal entry site (IRES) and a green fluorescent protein (GFP) sequence (Figure 4). This sequence allowed for translation of GFP independent of the translation of the suicide gene and was used to sort the transduced cells and normalize based on the rate of transcription. G816, stem-like glioblastoma cells, and U87MG, differentiated glioblastoma cells engineered to express EGFRvIII, were transduced and selected using fluorescence activated cell sorting. We decided to not use the U87MG engineered lines because we were not getting the same PEST stabilization using TMZ as we were getting with G816 cell lines. Following transduction and selection, expression of the suicide gene was assessed. TMZ induced increased expression of the suicide gene (measured by increased FLAG signal), confirming that cells were successfully transduced and that stability of the protein was regulated by DNA damage (Figure 5).

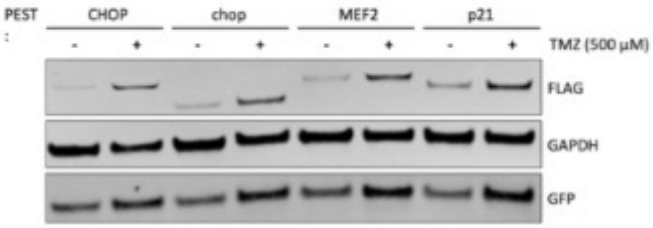

Figure 5. Through the use of western blotting, we were able to show the increase in FLAG-tagged HSVtk in the suicide gene cells treated with temozolomide versus those treated with DMSO (control). G816 (glioma-initiating cell line) cells were transduced with retrovirus carrying each of the four suicide genes. Different PEST domains confer varying baseline and TMZ-induced stabilities.

PEST Stabilization

To determine if p38 was responsible for the observed upregulation of HSVtk stability following TMZ treatment, engineered cells were treated with SB203580, a p38 inhibitor (Figure 6). There was a decrease in the TMZ dosage used from the blot shown in Figure 5, because with 500 μM TMZ and the additional p38 inhibitor there was too much cell death to measure protein stability. TMZ promoted protein stabilization due to the increase in FLAG seen between the control and the TMZ treated cases. In all of the cell lines there were decreases in FLAG in the TMZ and p38 inhibitor treated cases compared to the TMZ only treated cases. This result suggests that the suicide genes were stabilized

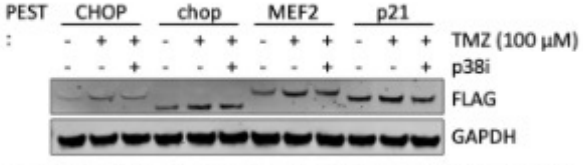

Fig 6. G816 cells expressing each of the four HSVtk constructs were grown for a day and then treated with DMSO, with or without TMZ (100 μM), and with or without SB203580 (p38 inhibitor, 10 μM) for three days.

through phosphorylation by p38 .

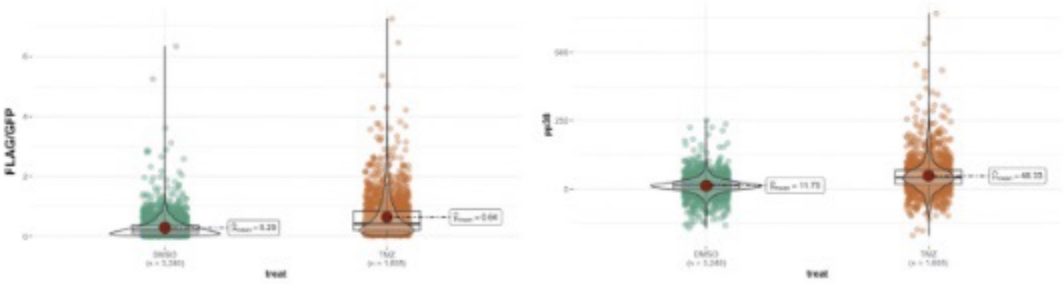

Figure 7. G816-chop lysates treated with or with out TMZ (100uM) for three days. FLAG was normalized by GFP to make sure that we were specifically looking at only cells that were GFP positive and contained the suicide gene.

We then moved to the use of flow cytometry to measure protein stability. This decision was made due to inconsistent results we were seeing at the bulk level which is what is measured using western blots. This method allows for single cell data to be collected and filtered for only GFP-positive cells. Also to note, we decided to use only the short chop cells for experimentation as those cells showed the greatest signal in western blots. Using flow cytometry, we measured FLAG, p-p38, and GFP.. Flow cytometry demonstrated an increase in FLAG with the treatment of TMZ, and an increase in phosphorylated p38 which would lead us to believe that the gene is a p38 regulated suicide gene (Figure 7). This is the same results we saw by western blot as well. These mean increases would show that the PEST domain is stabilized by TMZ compared to the control case.

We conducted a repeat flow cytometry experiment with the addition of the drug gossypetin, an MKK3 inhibitor. MKK3 is an upstream kinase of p38; thus, its inhibition should also inhibit p38. We needed to use the upstream kinase because direct inhibition of p38 drives its phosphorylation. If p38 is indeed responsible for the phosphorylation and stabilization of the suicide gene, its inhibition would be anticipated to drive a decrease in FLAG abundance. Unexpectedly, p38 phosphorylation was actually highest in the TMZ and gossypetin combination treated case (Figure 8). We also observed an unexpected decrease in FLAG following TMZ treatment and an increase following gossypetin treatment. Further troubleshooting is needed to understand these changes. We did also see a decrease in FLAG in the TMZ and gossypetin treated case yet we still saw an increase in phosphorylated p38 in this experiment. Thus, the relationship between p38 activity and PEST stabilization remains a question, and will require further troubleshooting to characterize.

Synergy of Combination Treatments

To determine if our suicide gene could be used synergistically with TMZ, we measured cell death using flow cytometry to measure To-Pro-3. Figure 9 shows the results of the experiment, and it shows the mean of three replicates with the standard deviations indicated. We

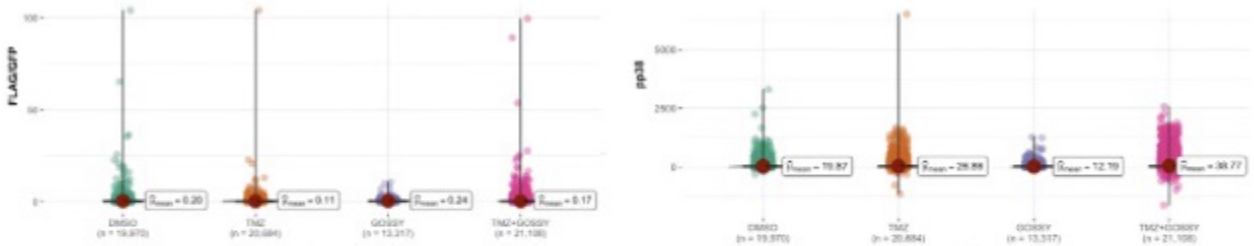

Figure 8. G816-chop lysates treated with or with out TMZ (100uM) and with or without gossypetin (10uM) for three days. FLAG was normalized by GFP to make sure that we were specifically looking at only cells that were GFP positive and contained the suicide gene.

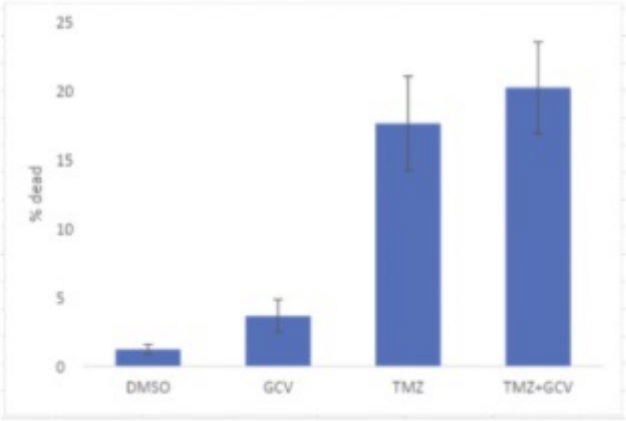

Figure 9. G816-chop lysates pretreated with or with out TMZ (300uM) for two day and with or without GCV (100uM) for three days.

attempted to try cotreatment, however we saw cell death protection. The reason for a pretreatment of TMZ followed by GCV is due to the mechanism of actions of each. Incorporation of phosphorylated GCVr into replicating DNA causes S-phase arrest; however, the cells are able to repair their DNA and move onto the next cell cycle phase (12). TMZ causes G2 arrest in which some cells will die and some cells will be able to complete mitosis (11). Due to GCV functioning in the S phase, the available HSVtk will phosphorylate the GCV before being able to accumulate due to PEST stabilization through temozolomide which would lead to protection from cell death (11). Despite pretreating with temozolomide, we did not observe a synergistic increase in cell death with the addition of GCV. Further optimization of timing and/or dosage is necessary.

Discussion

The main goal of this thesis was to design a suicide gene that uses a novel kinase pathway to synergize with chemotherapy to be a treatment method. We were able to successfully design four HSVtk fusion proteins with stability that was post-translationally controllable in response to DNA-damage driven stress. Though we were not able to definitively determine the kinase(s) responsible for suicide gene stabilization, we were able to demonstrate that temozolomide treatment increased stability, meaning this approach may be able to synergistically combine traditional chemotherapy with suicide gene treatment.

Remaining Challenges

There appeared to be some unusual results in the flow cytometry experiment shown in Figure 8, so we need to revisit the steps taken while conducting the experiment and redo the experiment as it could be a one-off result. We overall had issues with reproducibility that needs to be addressed and troubleshooting needs to occur. If the results hold true we would need to explore other kinase pathways as being responsible for stabilizing the PEST domain. One alternate theory is that JNK, another stress-induced kinase pathway, is stabilizing the PEST domain by phosphorylating p-cJun in a similar manner we theorized p38 would.

There also needs to be further troubleshooting in the GCV and TMZ combination synergy experiment. We may need to increase the pretreatment with TMZ to three days to allow for that max amount of HSVtk accumulation. Further screen tests are needed to determine what the issue may be.

Materials and Methods

Cell Culture

Cells were maintained in a cell culture incubator at 37 °C ,with5% CO_2. Glioblastoma-initiating cells (G816) were cultured nonadherently in neurobasal (Thermo Fisher Scientific) supplemented with penicillin/streptomycin, L-glutamine, B-27 minus vitamin A, and N2 supplements (Gibco). For experiments cells were plated in 6-well plates on Matrigel.

Western Blot

Cells were cultured and treated as appropriate for the experiment. After the appropriate timing cells were collected and cell lysates were prepared using a lysis buffer composed of protease inhibitor cocktail, phosphatase inhibitor 3, phosphatase inhibitor 2, and phenylmethylsulfonyl fluoride. These lysates were clarified using centrifugation at 20,000xg for 10 minutes

at 4 °C. Once lysates were collected, a micro-bicinchoninic (BCA) assay (Thermo Fisher Scientific) was run to determine protein concentrations needed for the blot. Once the concentration was determined cell solutions were made with about 20 micrograms of denatured total protein per sample and was loaded on 4-12% gradient polyacrylamide gels (Thermo Fisher Scientific) and transferred to 0.2 micrometer nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies that were diluted in Intercept Blocking Buffer (IBB, LI-COR) at a dilution of 1:1000 overnight at 4 °C. The membranes were then washed using 0.1% Tween-20 in PBS and incubated with secondary antibodies diluted in 1:10,000 in IBB for 2 hours at room temperature. The membrane was washed using PBS-Tween and imaged using the LI-COR Odyssey CLx system.

Flow Cytometry

Cells were cultured and treated as appropriate for the experiment. After the appropriate timing cells were collected and fixed using 4% paraformaldehyde for 15 minutes on ice. They were then permeabilized in 0.25% Triton-X-100 for 10 minutes at room temperature. Cells were then labeled using primary antibodies for one hour at 4 °C followed by secondary antibodies for 30 minutes at room temperature. Cells were analyzed using a FACSCalibur at the UVA Flow Cytometry Core Facility.

End Matter

Author Contributions and Notes

S.A., W.S.H., and M.J.L. designed research, S.A. and W.S.H. performed research, analyzed data, and wrote the paper.

W.S.H. and M.J.L. have filed a provisional U.S. patent application (Ser. No. 63/321,586) related to the p38-dependent suicide gene outlined in this work.

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